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The Role of Dietary Nitrate in Regulation of Blood Pressure and Metabolism

Michaela Sundqvist

Karolinska Institutet

From the Department of physiology and pharmacology
Karolinska Institutet, Stockholm, Sweden

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By

Michaela Sundqvist

Principal Supervisor:

Professor Jon Lundberg
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Pharmacology

Co-supervisors:

Professor Eddie Weitzberg
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Anesthesiology and Intensive Care

Professor Mai-Lis Hellénius
Karolinska Institutet
Department of Medicine
Division of Cardiology

Opponent:

Professor Jeremy Spencer
University of Reading
Department of Food an Nutritional Sciences

Examination Board:

Associate Professor Agneta Andersson
Uppsala University
Department of Food Studies, Nutrition and Dietetics

Professor Jan Östergren
Karolinska Institutet
Department of Medicine

Professor Claes Frostell
Karolinska Institutet
Department of Clinical Sciences

“This comes as no surprise, we’re emptying our supplies. We’re only thinking of the size of the pile of shit to buy. Over and over, we take what we want to take even if the bond with nature will break…”

Raised Fist, Killing It

Till mina älsklingar Leah och Robert

ABSTRACT

A diet rich in vegetables is recommended for health in general and is also associated with lower blood pressure. Research suggests that inorganic nitrate present in leafy green vegetables could be a contributing factor for the favourable effect. Nitrate from the diet is reduced to nitrite by oral bacteria and further metabolised to nitric oxide (NO) in blood and tissues. The nitrate-nitrite-NO pathway is an alternative way of NO formation in addition to endogenous NO production by the NO synthases. NO is a potent signalling molecule involved in regulation of blood flow and metabolic function.

In subjects with pre-hypertension and hypertension (n = 231) the effect on blood pressure after supplementing with nitrate-rich vegetables or potassium nitrate for 5 weeks was compared to low-nitrate vegetables. Also, we examined the handling of nitrate among women and men. Nitrate-rich leafy green vegetables or a nitrate pill did not lower 24 h ambulatory blood pressure in this patient group. However, the renal handling of nitrate seemed to differ between women and men at basal conditions.

In another study we investigated if interruption of oral bacterial conversion of nitrate to nitrite could affect metabolic rate in healthy volunteers. In this cross over study, subjects used antiseptic mouthwash or placebo for 3 days followed by measurements of resting metabolic rate. There was no difference in resting metabolic rate after antiseptic mouthwash use.

Lastly, the role of an acidic milieu in the gastric lumen for any acute blood pressure effect of nitrite was studied. Healthy men were given a proton pump inhibitor or placebo pills prior to a bolus dose of nitrite. Interestingly, the blood pressure reduction caused by nitrite was blunted by the proton pump inhibitor indicating that a low gastric pH is required for bio-activation of orally administered nitrite.

The studies in this thesis address cardiovascular and metabolic effects after supplementing or blocking the nitrate-nitrite-NO pathway. They have generated novel insights about clinical application of dietary nitrate intake, sex differences in nitrate handling and bio-activation of nitrite.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Forskning visar att en kost rik på grönsaker kan sänka blodtrycket. Nitrat är ett ämne som finns naturligt i många grönsaker t ex rödbetor och gröna bladgrönsaker. För att nitrat ska bli aktivt behöver det omvandlas till nitrit och sedan kvävemonoxid (NO). Detta sker med hjälp av bakterier i munnen och enzymer i blod och vävnader. Ett lågt pH stimulerar NO bildningen. De senaste åren har flera studier visat att tillskott av nitrat sänker blodtrycket hos både friska och personer med högt blodtryck. I vår forskargrupp har vi undersökt om ett tillskott av ca 125 g gröna bladgrönsaker om dagen säker blodtrycket jämfört med grönsaker som innehåller väldigt lite nitrat. I studien ingick även en grupp som fick en tablett med samma mängd nitrat som bladgrönsakerna innehöll. På så sätt kunde vi studera om det var nitrat i bladgrönsakerna och inget annat som gav en eventuell effekt på blodtrycket. Resultatet från studien visade dock att gröna bladgrönsaker rika på nitrat eller en tablett med nitrat inte sänkte blodtrycket jämfört med grönsaker med lite nitrat. Vi såg däremot att det skiljer sig mellan kvinnor och män hur kroppen hanterar nitrat.

Tillskott av nitrat har även visat kunna minska kroppens behov av syre och energi både i vila och vid träning. I en studie undersökte vi vilometabolismen (hur mycket energi som behövs vid vila) och om den förändras om vi hämmar bakterierna i munnen som hjälper till med omvandlingen av nitrat. Detta gjorde vi genom att deltagarna fick gurgla en antibakteriell munsköljning i tre dagar och sedan jämföra med munsköljning som inte påverkar bakterierna i munnen (placebo). Efter varje 3-dagarsperiod av munsköljning mätte vi vilometabolismen. Den skilde sig inte åt mellan gångerna. Vi kunde däremot kontatera att deltagarna effektivt eliminerat munbakterierna med den antibakteriella munsköljningen.

Den bakteriella nitratreduktionen i munnen leder till relativt höga halter av nitrit i saliv. Vi undersökte betydelsen av magsäckens pH (surhetsgrad) för att vi ska kunna se en blodtryckseffekt av nitrit. Nitrit bildar NO i magen då pH är tillräckligt lågt. Personerna i studien fick en medicin som höjer pH i magsäcken alternativt en placebotablett dagarna före blodtrycksmätning. Deltagare fick sedan nitrit och därefter mätte vi blodtrycket under två timmar. Då deltagarna hade fått placebotablett gick blodtrycket ner av nitrit, men när de tagit medicin som höjt pH i magen förändrades inte blodtrycket. Detta visar att ett lågt pH i magen är nödvändigt för att nitrit ska kunna sänka blodtrycket.

Dessa studier har undersökt effekter av nitrat och nitrit på hjärta och kärl samt vilometabolism. De har bidragit till ny information om effekt på blodtrycket efter ett ökat nitratintag genom bladgrönsaker, hur hanteringen av nitrat skiljer sig mellan kvinnor och män och betydelsen av lågt pH i magsäcken för aktivering av nitrit.

LIST OF SCIENTIFIC PAPERS

I. A randomized clinical trial of the effects of green leafy vegetables and inorganic nitrate on blood pressure.

Michaela L Sundqvist, Filip J Larsen, Mattias Carlström, Matteo Bottai, John Pernow, Mai-Lis Hellénius, Eddie Weitzberg* and Jon O Lundberg*.

Am J Clin Nutr. 2020; 00:1-8. * Shared senior authorship

II. Nitrate and nitrite handling in hypertensive men and women.

Michaela L Sundqvist, Mattias Carlström, Jon O Lundberg and Eddie Weitzberg. (Manuscript)

III. Effects of antiseptic mouthwash on resting metabolic rate: A randomized, double blind, cross over study.

Michaela L Sundqvist, Jon O Lundberg and Eddie Weitzberg.

Nitric Oxide. 2016; 61: 38-44.

IV. Blood pressure-lowering effects of orally ingested nitrite is abolished by a proton pump inhibitor.

Marcelo F Montenegro, Michaela L Sundqvist, Filip J Larsen, Zhengbing Zhuge, Mattias Carlström, Eddie Weitzberg and Jon O Lundberg.

Hypertension. 2017; 69(1): 23-31.

CONTENTS

LIST OF ABBREVIATIONS

ADI	Acceptable Daily Intake
BP	Blood Pressure
cGMP	Cyclic Guanosine Monophosphate
COX	Cytochrome C Oxidase
DASH	Dietary Approaches to Stop Hypertension
DBP	Diastolic Blood Pressure
DINO	Dietary Nitric Oxide Study
eNOS	Endothelial Nitric Oxide Synthase
FMD	Flow Mediated Dilation
HNO_2	Nitrous Acid
HR	Heart Rate
iNOS	Inducible Nitric Oxide Synthase
N_2	Nitrogen Gas
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric Oxide Synthase
nNOS	Neuronal Nitric Oxide Synthase
PP	Pulse Pressure
PPI	Proton Pump Inhibitor
RMR	Resting Metabolic Rate
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
sGC	Soluble Guanylyl Cyclase
VSMC	Vascular Smooth Muscle Cells
XOR	Xanthine Oxidoreductase

1 INTRODUCTION

The use of inorganic and organic nitrate has an interesting and long history. Already in the fifth century alchemist and physician Tao Hongjing described the medical use of saltpetre (KNO_3). This inorganic salt was placed under the tongue to treat patients with chest pain (1). Over a thousand years later the organic nitrate nitroglycerin was discovered and its use in medicine begun. The Swedish researcher and entrepreneur Alfred Nobel could stabilize nitroglycerin and develop an explosive well known as dynamite. The discovery made him extremely wealthy, and he used his fortune to create the Nobel Prizes. It was later realized that men with coronary disease who worked in the dynamite factories had fewer episodes with angina during work than over the weekend (2). Nitroglycerin and other organic nitrates are still used today to treat angina. However, this thesis will focus on the medical use of inorganic nitrate - the very old practice of Tao Hongjing.

1.1 Nitric Oxide production

Today we know that both inorganic and organic nitrates mediate their principal effects through the generation of nitric oxide (NO). NO is a tiny free radical gas and the discovery of endogenous NO production and signalling awarded Robert Furchgott, Louis Ignarro and Ferid Murad the Nobel Prize in Physiology or Medicine in 1998 (3-5). The molecule that was previously termed EDRF (endothelium-derived relaxing factor) was now identified to be NO.

In addition to its vasodilatory properties, NO is inhibiting leucocyte adhesion, platelet aggregation and vascular smooth muscle cell (VSMC) proliferation (6-8). NO is also produced in cardiac muscle where it is involved in regulating contractility (9). Consequently, NO has a central role in the cardiovascular system and reduced NO production and bioavailability are linked to several cardiovascular diseases (10).

Endogenously, NO is formed from NO-synthases (NOSs); which use the amino acid L-arginine and molecular oxygen as substrates (11). The NOSs exist in three isoforms: neuronal NOS (nNOS, NOS-1), inducible NOS (iNOS, NOS-2) and endothelial NOS (eNOS, NOS-3). Endothelial NOS is expressed in the vascular endothelium and regulates vascular tone and integrity (10). Activity and expression of eNOS decline with aging and this is also linked to hypertension (12). Constitutively expressed nNOS and eNOS produce moderate amount of NO under physiological conditions while induction of iNOS can lead to production of large amounts of NO as part of the immune response (13).

In vascular smooth muscle cells (VSMCs) NO stimulates soluble guanylyl cyclase (sGC) resulting in enhanced synthesis of the second messenger cyclic guanosine monophosphate (cGMP) which activates intracellular protein kinase G (PKG) ultimately leading to relaxation of vascular smooth muscle (11). During hypoxia the oxygen-dependent L-arginine-NO pathway is less effective resulting in decreased NO production (10). Conversely, during oxidative stress NO bioavailability is also decreased partly due to scavenging of the NO radical by reactive oxygen species.

NO is highly reactive with other radicals and transition metals, such as those found in haem proteins (14). Thus, in blood and tissues NO has a very short half-life and rapidly (from milliseconds to seconds) oxidises to nitrite (NO_2^-) and nitrate (NO_3^-) and with higher oxygen concentrations the rate of NO oxidation increases (15).

1.2 The nitrate-nitrite-NO pathway

In 1994 two research groups independently showed that salivary nitrite could form NO in the stomach (16, 17). This was the first observation of an NO-synthase independent pathway for NO generation. The year after Zweier and colleagues could demonstrate non-enzymatic NO generation from nitrite in the ischemic heart (18). These observations eventually lead to a new research field where supplementation of inorganic nitrate and nitrite can be used to enhance NO generation in vivo.

Nitrate in blood and tissues derives from oxidation of endogenously produced NO and from the diet (19). Plasma levels can therefore vary greatly depending on these factors. Fasting nitrate and nitrite levels in humans are around 20-40 μM and 0.05-0.3 μM respectively (20). Endogenously produced nitrate is estimated to be around 60 mg/day (1 mmol/d) (21). Nitrate from food has almost 100 % bioavailability (22) and plasma levels will peak after 15-30 minutes after ingestion with a half-life of 5-6h (19).

Circulating plasma nitrate is actively taken up in the salivary glands (25 % of the systemic nitrate) and concentrated in saliva (23). In the oral cavity facultative anaerobic bacteria, mostly located to the posterior part of the tongue, reduce nitrate to nitrite (24). Identified bacteria species of importance for the oral nitrate reduction include Veillonella atypica, Veillonella dispar and Actinomyces odontolyticus, but several other species are also efficient nitrate-reducers (25). Because of the active uptake and reduction of nitrate, salivary nitrate and nitrite are 100-1000-fold higher than in plasma (26).

Once swallowed, nitrite is rapidly protonated in the acidic gastric lumen to form nitrous acid (HNO_2), which then decomposes to NO and other nitrogen oxides in the stomach (27). This NO formation from nitrite is dependent on the low gastric pH, but also further enhanced by reducing agents such as dietary polyphenols

and ascorbic acid (19, 28, 29). A considerable amount of nitrite formed in saliva passes the stomach and is effectively taken up in the small intestine and enters the systemic circulation (30). In blood and tissues a number of proteins and enzymes have been found capable of further reducing nitrite to NO for example deoxyhaemoglobin, deoxymyoglobin, xanthine oxidoreductase (XOR) and components of the mitochondrial respiratory chain (31).

Due to the enterosalivary circulation of nitrate described above and the continuous swallowing of nitrite-containing saliva, plasma levels of nitrite will increase within 30 min after nitrate ingestion and stay elevated for several hours (32). The half-life of nitrite itself is only 20-30 min but the levels remain elevated because of continuous uptake of the more long-lived nitrate in the salivary glands (30).

Diminishing oral bacteria using an antiseptic mouthwash or antibiotics can nearly abolish the nitrate reducing capacity and also blunt the increase in plasma nitrite after a nitrate load (32). When using antiseptic mouthwash for seven days, oral nitrite production was reduced by 90 % and plasma nitrite levels with 25 % compared to a control period (33) clearly showing the crucial role of oral bacteria in the nitrate-nitrite-NO pathway.

Circulating nitrate is eventually excreted by the kidneys. A study using ^{15}N-labeled nitrate showed that around 60 % of a nitrate dose is excreted in the urine within 48 h (21). Supplementing with 10 mg/kg sodium nitrate increased concentrations of nitrate in urine from 0.8 mM to 8.1 mM in 3 hours (30). Also, intake of 120g of nitrate-rich lettuce for 3 days after a nitrate free diet resulted in four times higher nitrate levels in urine (34).

In summary, research performed during the past two decades verifies that nitrate and nitrite from dietary sources and from oxidation of endogenously produced NO can be recycled back to bioactive NO (20). This Nitrate-Nitrite-NO pathway represents an alternative way of NO generation in mammals in addition to the classical NOS-dependent pathway.

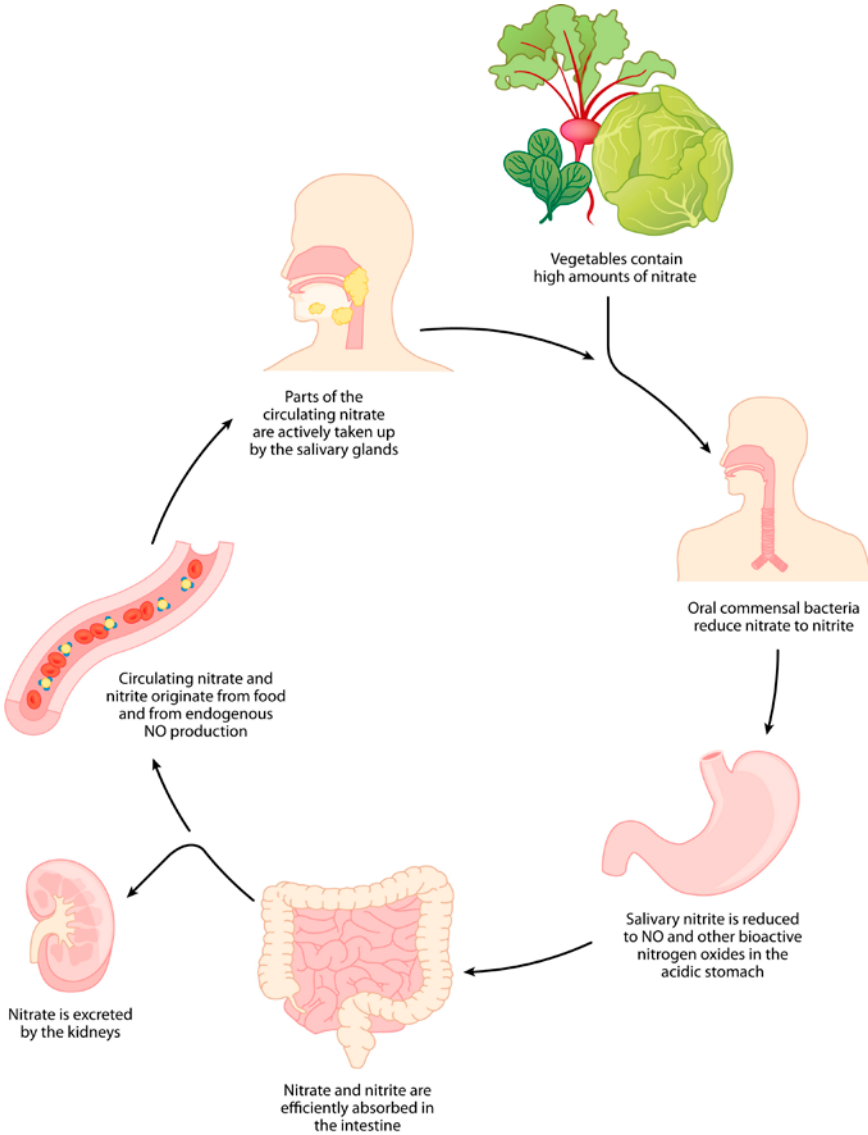

Figure 1. Nitrate metabolism. Inorganic nitrate is metabolised to NO and other bioactive nitrogen oxides in humans. Ingested nitrate is efficiently taken up in the gastrointestinal tract and enters the blood stream. Circulating nitrate is partly excreted by the kidneys, but 25 % is actively taken up by the salivary glands and secreted in saliva. In the oral cavity, salivary nitrate is reduced to nitrite by facultative anaerobic bacteria. In the acidic stomach swallowed nitrite is non-enzymatically reduced to NO and other bioactive nitrogen oxides. Remaining nitrite reaches the systematic circulation and is reduced NO and other nitrogen oxides by several pathways in blood and tissue. Weitzberg & Lundberg, Annual Reviews of Nutrition 2013 (19).

1.3 Dietary sources of nitrate and nitrite

Nitrogen (N_2) is the most abundant gas in the atmosphere. It's essential for all living organisms but to be biological active it needs to undergo fixation. Nitrogen-fixing bacteria convert nitrogen to ammonium (NH_4^+) and in a later step nitrifying bacteria oxidise ammonium to nitrite (NO_2^-) and then nitrate (NO_3^-). To complete the nitrogen cycle denitrifying bacteria eventually reduce nitrate back to nitrogen gas. Nitrate and nitrite are thereby a part of the nitrogen cycle and plays an important role in plant nutrition and function (35).

The main source of inorganic nitrate in the food is vegetables, which contribute with 60-80 % of the total intake (35). Table 1 show nitrate content in different vegetables. In certain meat-products (cured meat) nitrate and nitrite are added for colour, flavour and as preservatives, and this is responsible for 10-15 % of intake (36). Regarding nitrite 80-85 % stems from endogenous conversion of nitrate (37). Another major source of nitrate is the drinking water. In France it contributes with 14 % of the total nitrate intake and 22 % in the UK (35).

In plants nitrate plays an important role for growth with the highest concentration found in leafy green vegetables and herbs (35). The use of fertilizers has a big impact on nitrate levels in leafy vegetables (38). Seasonal variation also determines nitrate content showing higher nitrate during the darker months of the year (35, 39). UK grown lettuces showed a 24 % lower nitrate content in the summer than in the winter (40). Different handling of food and cooking methods can reduce nitrate content (35). Nitrate is highly water soluble and boiling vegetables for 15 min showed a significant decrease in nitrate content with a parallel increase in the boiling water (41).

A vegetarian diet has been shown to contain four times more nitrate compared to a non-vegetarian diet (42). Consumption of leafy green vegetables, which are the most nitrate-rich vegetables, has been shown to reduce the risk for cardiovascular events in several epidemiological studies (43-45). Hung and colleagues showed that an increment of one serving of green leafy vegetables per day was inversely associated with cardiovascular disease (45). Another study showed that one serving of fruit or vegetables was associated with a 6 % lower risk of ischemic stroke and the authors identified leafy green vegetables as one of the greatest contributors to the protective effect (43).

Table 1. Nitrate content in vegetables measured by the European Food Safety Authority (EFSA)[1] and by Lundberg laboratory[2]. ADI = Acceptable daily intake of nitrate.

High nitrate vegetables	Mean nitrate content[1] mg/100g	Mean nitrate content[2] mg/100g	% of ADI for a 70kg person[1]	% of average intake[1] in United Kingdom
Rocket/ Arugula	468	590	180	514
Spinach	106	158	40	116
Beetroot	138	443	53	152
Celery	110	514	43	121
Radish	97	143	38	107
Low-medium nitrate vegetables	**Mean nitrate content[1] mg/100g**	**Mean nitrate content[2] mg/100g**	**% of ADI for a 70kg person[1]**	**% of average intake[1] in United Kingdom**
Peas	3	0.1	1	3
Tomato	4	0.6	2	4
Capsicum	11	0.3	4	12
Cucumber	19	23	7	21
Carrot	30	11	12	29

1.4 Nitrate intake and potential risks

The acceptable daily intake (ADI) of nitrate and nitrite according the European Food Safety Authority (EFSA) is 3.7 mg/kg/day (35). Nitrate intake varies between countries and regions. In Europe the mean intake is 50-180 mg/day (37). The Mediterranean countries have a higher intake than in northern Europe. In the United Kingdom nitrate and nitrite intake is approximately 91 mg/day and in France 141 mg/day (35). Estimated intake in the United Stated is 40-100 mg/day (37).

The proposed toxicity of nitrate is linked to its conversion to the more reactive nitrite anion. Nitrite can induce methaemoglobinaemia and result in hypoxia in tissues with severe consequences (37). Background levels of methaemoglobin are around 1-3 %. At 10 % methaemoglobin a reduced oxygen transport is evident, at levels above 20 % cyanosis and hypoxia can occur and at 50 % methaemoglobin the outcome can be fatal (37). Lethal doses of nitrate in humans is estimated to be around 330 mg/kg body weight and nitrite is approximately 10-fold more toxic (35). However, a patient case study reported that an intake of 75 g sodium nitrate (833 mg/kg body weight) resulted in instant gastrointestinal symptoms but no peripheral cyanosis 4 h after intake (46). Infants younger than 3 months are more sensitive to methaemoglobin formation due to 40-50 % lower activity of the enzyme (NADH-cytochrome b5 reductase) which converts methaemoglobin back to haemoglobin (47).

For more than 50 years there has been extensive research in possible nitrosamine formation and cancer risk in relation to nitrate and nitrite intake. Animal studies on nitrate demonstrate low chronic toxicity and epidemiological studies do not

suggest that nitrate intake is associated with increased cancer risk (35). At the same time experimental human studies have shown increased levels of N-nitrosamines in the stomach after nitrate ingestion, using radioactive labelling confirming that the nitrogen originated from the given nitrate (48). Nitrite can form nitrosamines directly in the acidic stomach or in reactions catalysed by macrophages or bacteria (49, 50). The role of such nitrosamine formation is still not clear. A meta-analysis on gastric cancer in fact showed a reduced risk (RR 0.80) with dietary nitrate intake but an increased risk (RR 1.31) for nitrite (51).

Dietary sources of nitrite include both animal-based products and vegetables. However, over 80 % of the total nitrite exposure is a result of endogenous conversion of nitrate (35). Summarizing experimental and epidemiological research on nitrate and nitrite the International Agency for Research on Cancer (IARC) concluded in 2010 that there is "inadequate evidence in humans for the carcinogenicity of nitrate in food" and that there is "limited evidence in humans for carcinogenicity of nitrite in food" (52).

1.5 Nitrate and cardiovascular effects

In 2006 Larsen et al. demonstrated for the first time that intake of inorganic nitrate (0.1 mmol/kg for three consecutive days) lowered diastolic BP (-3.7 mmHg) in healthy volunteers (53). A few years later it was shown that 500 ml of nitrate-rich beetroot juice (22.5 mmol) decreased systolic BP by -10.4 mmHg, an effect coinciding with the peak in plasma nitrite levels. Interestingly, BP levels were still reduced even 24 h after ingestion of beetroot juice (54). A more recent study on 64 hypertensive patients measuring 24 h ambulatory BP after daily intake of 250 ml beetroot juice (6.4 mmol nitrate) for 4 weeks showed a reduction in SBP of -7.7 mmHg and for DBP -5.2 mmHg (55). In this study a placebo beetroot juice was used in which the nitrate anions had been selectively removed using a resin. Interestingly, it has also been shown that 800 mg nitrate from beetroot juice, spinach- and rocket-beverage lowered BP more than was seen with equal amount of sodium nitrate salt (56). In aggregate, numerous studies on healthy and hypertensive subjects have now demonstrated a BP-lowering effect of nitrate intake (57). The threshold for the effect among healthy individuals has been suggested to lie between 4-12 mmol of nitrate (58).

To investigate the role of endogenously generated nitrate on blood pressure Kapil and co-workers disrupted the nitrate-reducing capacity of the oral microflora with an antibacterial mouthwash for a week in healthy young subjects. This intervention together with a low-nitrate diet markedly reduced salivary and plasma nitrite levels with a concomitant increase in SBP by 3.5 mmHg (33). An increase in SBP (2.3 mmHg) has also been seen in hypertensive subjects using antibacterial mouthwash for three days (59). These results indicate that endogenously generated nitrate (from NOS) participates in regulation of blood pressure and that the oral microflora is necessary for this physiological function.

The endothelium has a crucial role in regulation of vascular tone, platelet activity, leucocyte adhesion and thrombosis (60). A major feature of endothelial dysfunction is reduced NO synthesis from eNOS and/or reduced NO bioavailability (42). A widely used measurement of endothelial function is flow mediated dilation (FMD) where a change in brachial artery diameter is measured in response to hyperemia after a standardized period of ischemia. Intake of sodium nitrate 12.7 mg/kg (0.15 mmol/kg) has been shown to improve FMD (61). In another study as little as 1mg/kg improved FMD compared to placebo, indicating that the amounts of nitrate ingested by people on a daily basis may contribute to endothelial integrity (29). Also, plasma nitrite levels which can reflect NOS activity (62) are positively correlated with flow mediated dilation (FMD) (63). A meta-analysis concluded that nitrate and beetroot supplementation was associated with beneficial effects on endothelial function, but with a reduced effect in older subjects and those with greater cardio-metabolic risk (64).

1.6 Nitrate and oxygen consumption

Energy expenditure in humans is most often measured by indirect calorimetry. The measurement is based on consumed oxygen and release of carbon dioxide and the assumption that metabolic heat production is reflected by the amount of oxygen consumed.

NO is involved in the control of mitochondrial respiration, and thereby energy regulation, by its reversible binding and inhibition of cytochrome c oxidase (COX) (65). COX is the terminal electron acceptor in the electron transport chain and the subunit COX IV-2 has been identified to strongly correlate to resting metabolic rate (RMR) (66).

In 2007 it was discovered that oxygen cost at submaximal exercise was reduced after supplementation with sodium nitrate (0.1 mmol/kg) (67). A few years later the same group showed that freshly harvested human skeletal muscle mitochondria became more efficient (generated more ATP per consumed oxygen) after intake of the same amount of sodium nitrate (68). This improvement correlated strongly to the reduction in whole body oxygen cost during exercise, suggesting that the effects of nitrate on the mitochondria underly the improvement in aerobic efficiency during exercise (68). Later, the same researchers showed a 4.2 % reduction in RMR in 13 healthy volunteers after 3 days of sodium nitrate 8.5 mg/kg/d (0.1 mmol/kg/d) compared to a placebo salt (NaCl) (69). These studies indicate that nitrate supplementation alters oxygen demand and energy expenditure.

2 AIMS

The overall aim of the thesis was to investigate the role of the nitrate-nitrite-NO pathway in regulation of blood pressure and metabolic rate. More specifically the aims were:

- To evaluate in a clinical trial the effects of leafy green vegetables on blood pressure in pre- and hypertensive subjects and elucidate if any such effect was due to their high nitrate content.
- To study levels of nitrate and nitrite in plasma, saliva and urine after nitrate restriction and examine if there was any difference between women and men.
- To investigate if nitrate, originating from endogenous NO production, affects resting metabolic rate and blood pressure in healty subjects.
- To study the role of gastric acidity on the acute cardiovascular effects of nitrite.

3 MATERIAL AND METHODS

A summary of material and methods is presented below. For a more detailed description, see the material and method sections for each scientific paper.

Study I: A randomized clinical trial of the effects of green leafy vegetables and inorganic nitrate on blood pressure

Study II: Nitrate and nitrite handling in hypertensive men and women

Study III: Effects of antiseptic mouthwash on resting metabolic rate: A randomized, double blind, cross over study

Study IV: Blood pressure-lowering effects of orally ingested nitrite is abolished by a proton pump inhibitor

3.1 Subjects

In study I pre-hypertensive and hypertensive subjects (n = 231, SBP 130-159 mmHg, age 50-70 years) living in Stockholm County were enrolled. Exclusion criteria were cardiovascular event the last 6 months, changed dose of anti-hypertensive medication the past 2 months, use of nitroglycerine, proton pump inhibitors or insulin as an antidiabetic treatment. A detailed list of exclusion criteria is found in the manuscript. In Study II women (n = 122) and men (n = 109) from Study I were investigated with regard to handling of nitrate and nitrite. In Study III healthy women (n = 17, age 23 ± 4 years) were enrolled. Study IV was performed with two protocols both on healthy men (n = 15, age 30 ± 7 years and n = 8, age 36 ± 16 years respectively). In Study III and IV subjects were not included if they were smokers or taking chronic medication.

3.2 Nitrate and nitrite supplementation

In Study I eligible subjects first entered a 2-week run in period and were instructed to eat two portions (≈ 125 g/day) of low-nitrate vegetables (cherry tomatoes, peppers, mini carrots and sweet corn) and exclude all other vegetables and legumes in their otherwise usual diet. After two weeks the subjects were randomized to one of three interventions on top of their usual diet: low nitrate vegetables + placebo pills (potassium chloride, twice daily), low-nitrate vegetables + nitrate pills (150 mg of nitrate in the form of potassium nitrate, twice daily) or leafy green vegetables (amount adjusted to contain 150 mg nitrate, twice daily) + placebo pills. The subjects were instructed to take a pill with their given vegetables as a complement to their lunch and dinner. High nitrate vegetables included leafy green vegetables corresponding to 300 mg of nitrate daily (≈ 125 g/day). The weight of low nitrate

vegetables was matched after each measurement. The nitrate content of vegetables used in the study was measured two times per semester to calculate daily portions of high nitrate vegetables and a matched amount of low nitrate vegetables.

Estimated nutritional content for the different vegetable groups was also calculated (Table 2). The daily portions of high-nitrate vegetables and low-nitrate vegetables have only minor nutritional differences as calculated from data bases. The difference in energy between high nitrate vegetables and low nitrate vegetables is around 40 kcal per day and a total of 1500 kcal for the intervention period of 5 weeks.

Table 2. Estimated nutritional composition in the daily dose vegetables given in the DINO study. RI = Recommended Intake for adult women and men. Nordic Nutrition Recommendation 2012.

Macro nutrients	**RI/day**	**High nitrate vegetables**	**Low nitrate vegetables**	**Percent (%) of RI**
kcal	-	16	59	-
H_2O (g)	1000-1500	110	99	11/10
Protein (g)	-	1.6	1.6	-
Fat (g)	-	0.2	0.3	-
Carbohydrates (g)	-	1.3	11.1	-
Fibre (g)	25-35	1.4	2.1	6/8
Micro nutrients	**RI/day**			
Vitamin A RE (µg)	700-900	125	200	18/29
Vitamin D (µg)	10-20	0	0	0/0
Vitamin E (mg)	8-10	0.3	1.4	4/18
Thiamine (mg)	1.0-1.4	0.1	0	10/0
Riboflavin (mg)	1.2-1.6	0.1	0.1	8/8
Niacin (mg)	13-19	0.6	1	5/8
Vitamin B_6 (mg)	1.2-1.5	0.1	0.2	8/17
Folate (µg)	300-400	84.4	41.7	28/14
Vitamin B_{12} (µg)	2	0	0	0/0
Vitamin C (mg)	75	10	66	13/89
Calcium (mg)	800	75	10	9/1
Phosphorus (mg)	600	37	43	6/7
Potassium (mg)	3100-3500	416	238	13/8
Sodium (mg)	2400	7	103	0.3/4
Magnesium (mg)	280-350	15	17	5/6
Iron (mg)	9-15	0.8	0.5	9/6
Zinc (mg)	7-9	0.58	0.41	8/6
Selenium (µg)	50-60	0.58	0.51	1/1

A validation of the nitrate pill and vegetable intake in terms of bioavailability was performed prior to the start of Study I. Plasma levels of nitrate and nitrite after intake of nitrate-rich vegetables or KNO_3 were evaluated in a pilot study before the study start. A cross over design was used in seven healthy volunteers. A baseline blood sample (0 h) was taken before a standardized breakfast (0.5 h) together with either nitrate-rich vegetables (150 mg NO_3^-) or KNO_3 (150 mg NO_3^-). Plasma nitrate and nitrite were measured by HPLC (ENO-20; EiCom) 4 h and 8 h after the baseline blood sample. Plasma nitrate and nitrite levels were not significantly different after vegetable intake compared to salt intake (Figure 2).

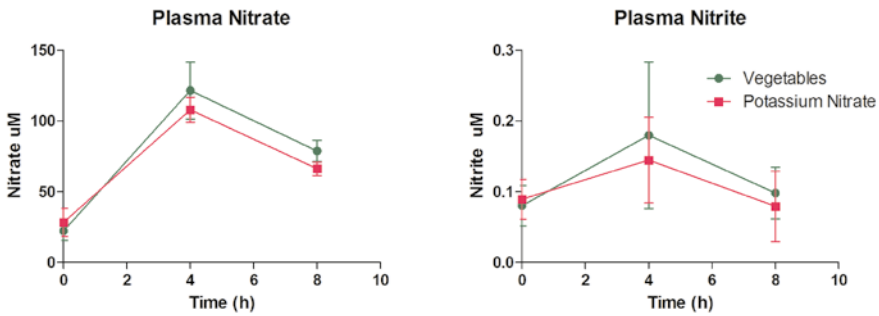

Figure 2. Plasma nitrate and nitrite were measured at baseline, 4 h and 8 h after intake of 150 mg NO_3^-. Data was analysed with 2-way repeated measures ANOVA followed by Bonferroni post hoc analysis. There was no significant difference in plasma nitrate or nitrite after vegetables intake compared potassium nitrate intake. Data is presented as mean ± SD.

In Study III the subjects were instructed to only eat low-nitrate vegetables and avoid cured meat 3 days prior to the test day. Study IV was performed in two parts. In part one the subjects (were instructed not to eat any vegetables or cured meat the day before and during the test day. On the test day they got a bolus dose of 0.3 mg/kg sodium nitrite. In the second part of the protocol nitrite was infused intravenously at 3 different rates (1, 10 and 30 nmol/kg/min) for ten minutes at each dose.

3.3 Study procedures

The study procedure for Study I is presented in Table 3. The study included 10 visits in total for the subjects running over a period of minimum 7 weeks. In Study II data (blood-, saliva samples and 24 h urine collection) was extracted from screening visit 2 and from visit 7 in the placebo group.

In Study III the subjects were randomised to rinse/gurgle their mouth for 1 min with placebo or chlorhexidine-containing mouthwash 3 times a day for 3 days prior

to measurements. The study had a cross over design and the 3-day intervention period was separated by 28 days. On the morning of the third day of intervention the subjects were instructed to attach a 24 h blood pressure monitor, collect urine and record their food intake. The following day the subjects returned after > 12 h fasting to measure body temperature, RMR, exhaled NO and office BP. Also, blood- and saliva samples were taken and a nitrate reducing ability test was performed.

In the first part of Study IV the subjects were pre-treated with placebo or esomeprazole (40 mg) 16, 8 and 1 hour before sodium nitrite intake. After nitrite intake, repeated BP measurements and blood sampling were performed during 2 hrs. In the second part, BP was measured immediately after each nitrite infusion period using a sphygmomanometer. A blood sample was collected after the final nitrite infusion period.

Table 3. An overview of the protocol for each study visit in Study I.

Screening visit 1
Informed written consent
Major eligibility questions
Medical history
Blood pressure x 3, mean of the two last. If SBP is 135-169 mm Hg and DBP < 110 mm Hg proceed to next visit.
Screening visit 2
Blood pressure x 3, mean of the two last. If SBP is 130-159 mm Hg and/or
DBP < 110 mm Hg participants are included in the study.
General lifestyle questionnaire
Instructions for 3d dietary record
Height and weight
Body composition measurement
Saliva and tongue test
Run-in period (2 w)
Distribute materials and information for 24-h urine collection
Hand out low nitrate vegetables
Randomization visit – 1st test visit
Urine collection 24h
Blood pressure x 3, mean of the two last.
Blood sample
Saliva and tongue sample
Weight and body composition
Attach 24h BP monitor and 24h activity record
Randomise

Intervention visit 1-3
Blood pressure x 3, mean of the two last.
Saliva sample
Weight and body composition
Hand out vegetables
Intervention visit 4
Blood pressure x 3, mean of the two last.
Saliva sample
Weight and body composition
Hand out vegetables and pills
Instructions for 2nd 3d food record and urine collection
Intervention visit 5 – 2nd test visit
Urine collection 24h
Blood pressure x 3, mean of the two last.
Blood sample
Saliva and tongue sample
Weight and body composition
Attach 24h BP monitor and 24h activity record
General lifestyle questionnaire

3.4 Blood pressure measurements

Ambulatory blood pressure was performed using WatchBP® O3 (Microlife Corporation, Switzerland) validated by the European Society of Hypertension (ESH) for clinical, ambulatory and self-measurement. In Study I and II the monitor was programmed for one reading every 30 min for 24 h and in Study III every 30 min between 6 am -10pm and one reading every 1h between 10 pm-6 am. WatchBP® O3 Analyser Software was used to download readings and produce mean day- and night-time BP. In all studies clinic and office BP was measured using an Omron M10-IT (Omron Corporation, Japan). After a 5 min rest in a quiet room in a sitting position, three measurements with 1.5 min intervals were taken in the upper arm (lower arm in an armrest, 90 degrees angle). The second and third readings were averaged to determine a mean clinic BP. All BP observers were blinded to the group assignment and participants were blinded to their BP data.

3.5 Measuring nitrate in vegetables

The first step involves finely chopping the vegetables using a mixer and then adding distilled water (3 times the weight). The vegetable solution is poured into Falcon tubes and stored in 56 C° water for 45 min (allowing the water soluble nitrate to

equilibrate), followed by centrifugation (10 000 x g for 15min). The supernatant was pipetted to Eppendorf tubes and stored at -80 C°. Concentrations of nitrite and nitrate were determined by chemiluminescence after reductive cleavage and subsequent release of NO into the gas phase as described previously (30).

3.6 Blood-, saliva and urine sampling

Blood samples were taken via standard venepuncture and using vacuum test tubes in which nitrite free EDTA solution (250 mmol/l; 8ul/ml blood) had been added. For measurements of plasma nitrate and nitrite the blood samples were centrifuged for 10 min, at 4 C° at, 2000 rpm and 0.5 ml aliquots plasma were pipetted into Eppendorf tubes (1.5 ml). Saliva samples (0.5ml) were collected in 1.5 ml Eppendorf tubes and frozen immediately. Urine was collected in sterile containers and the total time of the urine collection was documented by the subjects. The subjects were instructed to store the containers in the fridge when possible. Total urine volume was registered and aliquoted in Eppendorf tubes (0.5 ml). Plasma-, saliva- and urine samples were stored at -80 C° until nitrate and nitrite levels were measured using a HPLC system (ENO-20; EiCom) as previously described (32, 70). Nitrate and nitrite measurements were performed at a laboratory at the Department of Physiology and Pharmacology. Clinical laboratory tests (plasma lipids, glucose, electrolytes etc.) were measured in the central laboratory at Karolinska University Hospital.

3.7 Measuring resting metabolic rate

Resting metabolic rate (RMR) was measured using an indirect calorimetry system with a ventilated hood (Jaeger Oxycon Pro). The test started with a 15 min rest in a quiet dark room, after which RMR was recorded for 30 minutes. The lowest oxygen uptake monitored for 10 consecutive minutes was used as a measure of RMR. The test was performed at a separate visit prior to the intervention to familiarize the subjects with the method. Body temperature was measured with an ear thermometer (Braun ThermoScan) at each visit.

3.8 Statistics

In Study I a pre study power calculation was performed and a sample size of 60 subjects per group was estimated to provide > 80 % power to detect a 3 mmHg difference in change in SBP between groups using unpaired comparison. Between-group differences in change in BP were analysed with one-way ANOVA followed by Bonferroni post-hoc analysis. Within group comparison in BP was analysed with two-way repeated measures ANOVA also followed by Bonferroni post-hoc analysis. The weekly SBP and DBP were analysed with Linear Mixed Model.

Data are presented as means ± SD. D'Agostino & Pearson omnibus test was used to determine normal distribution of data. Tests were considered significant at a p value < 0.05. Statistical analysis was performed in Prism 5 and 6 software (Graph Pad) and STATA version 13 (StataCorp, Collage Station, TX, USA).

In Study II normally distributed data was analysed with paired or unpaired t-tests. Non-normally distributed data was analysed with Wilcoxon matched-paired signed rank test or Mann Whitney tests. Data are presented as means ± SD or median and 25-75 percentile.

In Study III paired t-test was used to compare mouthwash and placebo treatments. Data are presented as means ± SD or median and 25-75 percentile.

In Study IV paired t-tests, 2-way ANOVA using Bonferroni post hoc analysis or 1-way ANOVA followed by Dunnett multiple comparison tests were used. Data are presented as means ± SEM.

4 RESULTS AND DISCUSSION

4.1 Study I

4.1.1 Adherence

In Study I the adherence to the protocol was evaluated with self-reported intake, blood and urine samples. The self-reported compliance to the vegetable and pill intake was > 97 % and > 98 % respectively. In accordance with these results, plasma nitrate increased significantly in the intervention groups while in the placebo group it remained unchanged compared to baseline levels (Table 4). However, only in the potassium nitrate group did the increase in plasma nitrite reach significance. The modest increase in nitrite levels in plasma could be explained by the time point of the sampling which was after an overnight fasting, i.e. around 12 h after the last nitrate intake. Saliva nitrate and nitrite increased in the groups receiving nitrate. Consistent with plasma and saliva, urinary nitrate excretion was similar (254 mg and 303 mg) in the groups receiving nitrate and close to the total amount nitrate given, confirming excellent compliance.

Table 4. Nitrate (plasma, saliva and urine) and nitrite (plasma and saliva) before and after interventions. Within group comparison was analysed with repeated measures two-way ANOVA followed by Bonferroni post-hoc analysis. [1]Significantly different from pre value $p < 0.05$. [2]Significantly different from pre value $p < 0.0001$. All values are presented as means ± SDs.

Groups	Placebo		Potassium Nitrate		Leafy Green Vegetables	
	Pre	Post	Pre	Post	Pre	Post
Nitrate						
Plasma, µmol/L	31.2 ± 18.9	32.0 ± 20.2	30.6 ± 12.7	105 ± 52.4[2]	30.4 ± 11.6	114 ± 57.9[2]
Saliva, µmol/L	286 ± 449	315 ± 596	412 ± 432	1802 ± 1633[2]	322 ± 353	1557 ± 1463[2]
Urine, µmol/L	513 ± 296	526 ± 320	582 ± 291	2593 ± 1175[2]	526 ± 297	2843 ± 1167[2]
Excreted, mg/d	51.3 ± 23.1	53.0 ± 43.5	58.1 ± 30.1	254 ± 86.7[2]	54.0 ± 29.7	303 ± 127[2]
Nitrite						
Plasma µmol/L	0.34 ± 0.25	0.33 ± 0.28	0.36 ± 0.22	0.46 ± 0.38[1]	0.39 ± 0.33	0.48 ± 0.45
Saliva, µmol/L	147 ± 192	158 ± 160	190 ± 185	604 ± 633[2]	186 ± 214	499 ± 648[2]

4.1.2 Blood pressure

In this randomized, placebo-controlled clinical trial the primary outcome measure was difference in change in 24h ambulatory SBP (Figure 3). The numerical change in SBP was -0.6 ± 6.2 mmHg in the placebo group, -1.2 ± 6.6 mmHg in the potassium nitrate group and -0.5 ± 6.6 mmHg in the leafy green vegetables group (all non-significant). Also, there was no significant difference in delta SBP between the groups. Clinic SBP taken before and in the last day of the intervention period showed a decrease in SBP in the potassium nitrate group (-4.3 ± 11.6 mmHg, $p < 0.001$) but not in the leafy green vegetables group (-1.7 ± 11.2 mmHg, $p = 0.181$) or the placebo group (-1.3 ± 9.1 mmHg, $p = 0.227$) (Figure 3).

In a recently published meta-analysis the effect of nitrate supplementation on BP has been evaluated with the conclusion that SBP is reduced by dietary nitrate intake (-4.1mmHg, $p < 0.001$) in clinic settings, but not with home- or ambulatory SBP measurements (57). Ambulatory BP measurements has been pointed out as a better predictor of hypertension mediated organ damage than office BP (71). Also, ambulatory BP measurements can identify white coat hypertension which is more common in older people and those with grade 1 hypertension (72). On the other hand a clinic BP performed in accordance with guidelines on the same time-point of the day could be an easier tool to detect small (3-4 mmHg), but clinically relevant changes in SBP. In Study I night-time ambulatory SBP decreased from 123 mmHg to 121 mmHg in the group receiving potassium nitrate ($p = 0.041$), suggesting that a more standardized measurement, during night-time when lying down, is needed to see minor changes in SBP. However, the largest previous study on the effect of nitrate in hypertensive subjects showed an impressive reduction in 24 h ambulatory BP by -7.7/-5.2 mmHg and in clinic BP by -7.7/-2.4 mmHg after intake of ≈ 400 mg nitrate (55). In this study performed by Kapil and colleagues baseline ambulatory SBP was 148 and 149 mmHg in the placebo and intervention group, respectively and thereby nearly 20 mmHg higher than the mean baseline ambulatory SBP (131 mmHg) in Study I. The difference in starting BP could be an explanation of the contrasting study outcomes, although baseline BP have not been associated with effect size in the earlier mentioned meta-analysis on dietary nitrate and BP (57).

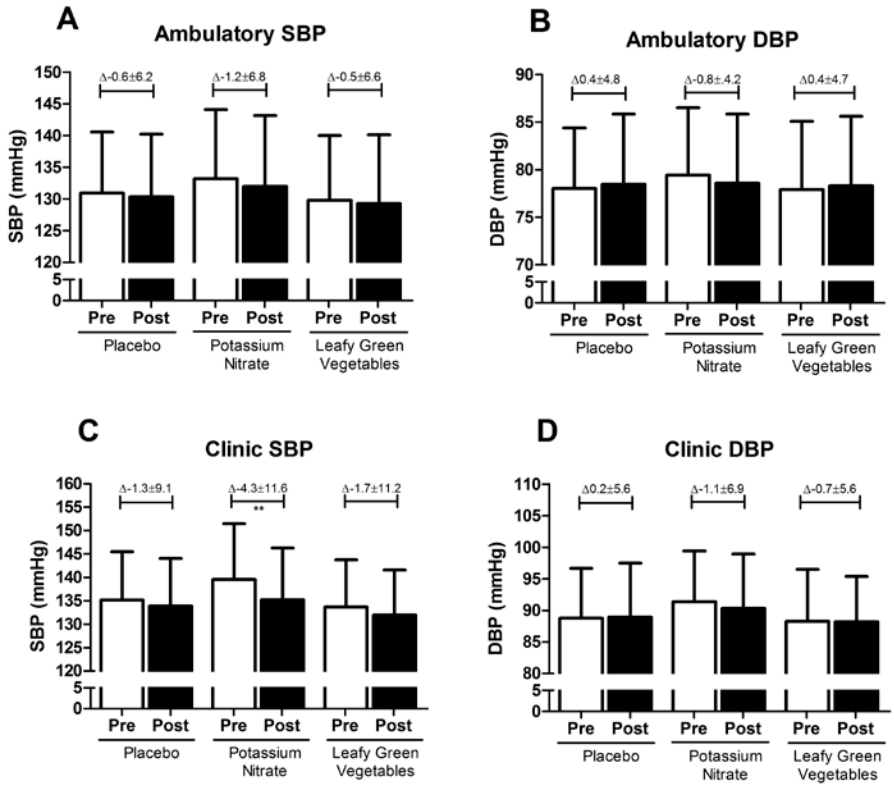

Figure 3. Ambulatory BP (A, B) and Clinic BP (C, D) before and after intervention. There was no difference in difference in change in between group comparisons using 1-way ANOVA with Bonferroni correction. Within group comparisons were performed using 2-way ANOVA followed by Bonferroni post hoc analysis. Clinic SBP (C) was significantly reduced in the potassium nitrate group after the intervention.

The absence of a BP-lowering effect in Study I could be due to the amount of nitrate given daily. The dose of 300 mg/day was carefully chosen to be achievable trough a normal diet and resulted in a mean of 4.0 mg nitrate/kg daily for the study subjects (4.4 mg/kg for women and 3.6 mg/kg for men). This means that the female subjects slightly exceeded EFSAs upper limit which is 3.7 mg nitrate/kg/day (35). However, there was no correlation with dose/kg and effect on BP and the threshold is suggested to be 250 mg nitrate for a BP effect of nitrate (58).

The effect of nitrate-rich leafy green vegetables on blood pressure has until today been investigated in two clinical trials other than Study I. Ashworth et al. (73) showed in a cross over study a -4 mmHg decrease in clinic SBP in normotensive healthy female volunteers (n = 19, mean age 20 years) after 1 week of high nitrate

vegetables (mainly leafy greens). Compared to our study there was a difference in age and baseline BP. Also, the participants were instructed to consume the vegetables 2.5-3 h before blood pressure measurement – that is when nitrite levels are peaking (32, 54). Indeed, the subjects had almost four-fold higher nitrite levels than the subjects in Study I. This procedure could be viewed as a more acute experiment looking at BP compared to Study I. Moreover, 8 out of 19 subjects had no effect or an increased SBP after leafy green vegetable which makes it hard to draw firm conclusions. The second study performed by Bondonno et al. had more similarities with Study I (74). The amount of nitrate supplemented form leafy green vegetables was calculated to contain 300mg (the main source was frozen spinach) and the age group was 38-70 years old. In this controlled cross over study (n = 38) they measured BP in three ways (ambulatory, clinic and home BP) but could not detect any changes with any of the methods when comparing 1 week of high-nitrate vegetables with 1 week of nitrate restriction. The time-point of intake in relation to BP measurement seems to be crucial to detect an effect of leafy green vegetables. In Study I a small but significant decreases in SBP was seen in the potassium nitrate group (Figure 3, C) and not in the leafy green vegetable group and it cannot be excluded that some other compound present in leafy green vegetables attenuates the effect of nitrate.

4.1.3 Sex differences

An interesting un-published observation in Study I was that females exhibited a significant drop in 24h ambulatory SBP in the potassium nitrate group (-2.5 mmHg, $p = 0.01$), but no changes were seen in the leafy green vegetables group (-0.3 mmHg) or the placebo group (-1.3 mmHg, Figure 4). Also, clinic SBP in women was reduced by -5.5 mmHg ($p = 0.004$) in the potassium nitrate group. There was no significant reduction in clinic or ambulatory BP in men (Figure 4).

In the study by Ashworth et al. mentioned earlier the young women had a reduction in SBP despite low baseline BP (73). However, in a study by Kapil and colleagues on sex differences related to the nitrate-nitrite-NO pathway, men had a greater numerical (non-significant) decrease in SBP (-6 mmHg) compared to women (-2 mmHg) (75). Oestrogen has been shown to have a regulatory role on endogenous NO production (76). The women in Study I were post-menopausal and it is possible that hormonal changes influence the BP response in women of different ages but not in men. Any possible sex differences in the effect of nitrate on BP needs to be elucidated further.

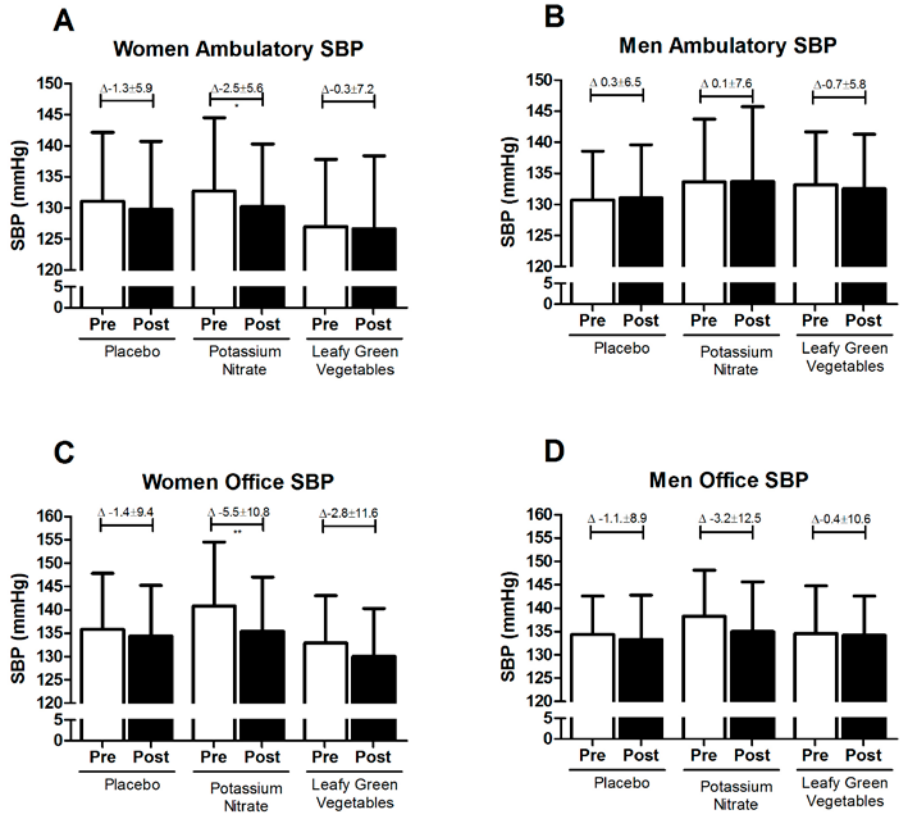

Figure 4. Ambulatory SBP in women (A) and men (B) and clinic SBP in women (C) and men (D) before and after intervention. Within group comparisons were performed using 2-way ANOVA followed by Bonferroni post hoc analysis. Ambulatory SBP and clinic SBP in females (A, C) were significantly reduced in the potassium nitrate group.

4.2 Study II

4.2.1 Sex differences in nitrate excretion

Nitrate handling was investigated in women (n = 122) and men (n = 109) after two weeks of dietary nitrate restriction. The data in Study II were extracted from the participants in Study I. There was no difference in nitrate and nitrite levels in plasma or saliva between women and men (Table 5). However, plasma nitrite was close to significantly higher in women (0.38±0.24 in women and 0.34 ± 0.30 in men, $p = 0.09$). Nitrate concentrations in urine, excreted nitrate as well as nitrate clearance were all lower in women ($p < 0.0001$) as well as the fractional excretion of nitrate ($p = 0.0013$). This was apparent despite no difference in volumes of urine excreted during 24 h (1763 ± 642 ml/24h and 1791 ± 648 ml/24h in women and men respectively, $p = 0.95$) or estimated glomerular filtration rate (80.1 ± 11.5 and 80.1 ± 8.4, $p = 0.28$).

Circulating nitrate is commonly used as a surrogate marker of endogenous NO production but is not reliable when dietary nitrate intake is not controlled for. Study I had a relatively long and controlled period of low nitrate intake which gave us an opportunity to study endogenous NO production and renal handling of nitrate in depth.

In the study by Kapil and colleagues mentioned earlier on sex differences in the nitrate-nitrite-NO pathway they reported higher nitrite levels in plasma, urine and saliva in women, but similar nitrate levels in all these matrices (75). They also performed analysis of oral nitrate reductase activity by holding a fixed volume of potassium nitrate in the mouth for 5 min which resulted in higher nitrite conversion in women, but the authors found no sex differences in the composition of the oral microbiome. Also, after a given nitrate dose of 500 mg the fold increase in plasma nitrite was significantly higher in women compared to men. As mentioned above plasma nitrite was showing a trend towards higher levels in women in our study, which is partly in line with Kapil and colleagues, but salivary nitrite was not different (167 ± 196 μM in women and 182 ± 192 μM in men, $p = 0.42$).

Table 5. Nitrate (plasma, saliva and urine) and nitrite (plasma and saliva) after two weeks of low-nitrate diet. $FE_{nitrate}$; renal fractional excretion of nitrate, $C_{nitrate}$; renal clearance of nitrate. Saliva ratio is salivary nitrite/salivary nitrate. Normally distributed data is analysed with unpaired t-test and non-normally distributed data with Mann-Whitney test. Values are presented as means ± SDs and ratio as median (Q1-Q3).

Nitrate	Women (n =122)	Men (n=109)	Significance
Plasma (μM)	31.8 ± 13.3	29.6 ± 16.2	p = 0.10
Saliva (μM)	344 ± 380	391 ± 462	p = 0.75
Urine (μM)	469 ± 290	619 ± 281	p < 0.0001
Excretion (mg)	47.2 ± 28.3	62.5 ± 25.0	p < 0.0001
$FE_{nitrate}$ (%)	17.7 ± 7.70	20.8 ± 6.41	p < 0.01
$C_{nitrate}$ (ml/min)	17.7 ± 8.19	26.1 ± 10.27	p < 0.0001
Nitrite			
Plasma (μM)	0.38 ± 0.24	0.34 ± 0.30	p = 0.09
Saliva (μM)	167 ± 196	182 ± 192	p = 0.42
Saliva Ratio	0.43 (0.29-0.84)	0.46 (0.28-0.95)	p = 0.89

In a study by Forte et al. on NO generation in healthy women and men (age 20-42 years), $_{L}$-[^{15}N]$_2$-guanidino arginine was administered intravenously and excreted urinary ^{15}N nitrate was measured. Women excreted significantly more N^{15} nitrate and it was positively correlated to 17β-estradiol (77), indicating higher endogenous NO formation by NOS in women. Hormonal differences are one reason for discrepancies in nitrate excretion and several studies have shown that estradiol treatment increases eNOS activity (76, 78). In Study II females were post-menopausal which could explain the contrasting results and consequently we suggest a lower endogenous NO generation in women than in men in this age group. Moreover, a study in untreated hypertensive subjects using the same method as described above showed lower ^{15}N nitrate excretion in hypertensive subjects compared to healthy controls (79). In both studies using the L-arginine isotope there was an inverse correlation between excreted nitrate and mean arterial blood pressure. We could not confirm these findings in our study.

In Study II we saw a clear difference in nitrate excretion, nitrate clearance and fractional excretion of nitrate between sexes indicating different renal handling of nitrate in women and men, at least in our cohort. Regarding the underlying mechanisms, any differences in uptake of nitrate in the gastro-intestinal tract, storage ability in different tissues or the renal handling are still not clear.

4.2.2 Time aspect of nitrate restriction

We wanted to investigate if more prolonged dietary nitrate restriction would alter handling of nitrate and nitrite. The placebo group from Study I (n = 78) were on a low nitrate diet for 7 weeks in total, i.e. 5 additional weeks after the initial 2-week run in period. Table 6 show nitrate and nitrite levels in plasma, saliva and urine as well as the amount nitrate excreted after 7 weeks of a low nitrate diet among women (n = 42) and men (n = 36). The difference between women and men in urinary nitrate concentration, excreted nitrate, nitrate clearance and fractional extraction of nitrate was sustained for the additional 5 weeks of nitrate restriction. In addition, plasma nitrite reached significantly higher levels in women in line with the study on sex differences by Kapil et al. (75). As of today Study I is the longest intervention on effects of a low nitrate diet, although several shorter placebo controlled supplementation studies have reported unchanged levels of plasma nitrate over periods of 1-4 weeks in the control group (55, 73, 74).

Table 6. Nitrate and nitrite in plasma, saliva and urine after 7 weeks of low-nitrate diet. $FE_{nitrate}$; renal fractional excretion of nitrate, $C_{nitrate}$; renal clearance of nitrate. Saliva ratio is salivary nitrite/salivary nitrate. Normally distributed data are analysed with unpaired t-tests and non-normally distributed data with Mann-Whitney tests. Values are presented as means ± SDs and ratio as median (Q1-Q3).

Nitrate	**Women (n=42)**	**Men (n=36)**	**Significance**
Plasma (μM)	33.2 ± 12.2	32.0 ± 27.6	p = 0.06
Saliva (μM)	271 ± 270	364 ± 827	p = 0.79
Urine (μM)	445 ± 284	621 ± 336	p = 0.01
Excretion (mg)	40.9 ± 22.7	67.0 ± 56.4	p < 0.0001
$FE_{nitrate}$ (%)	15.8 ± 7.09	21.2 ± 6.53	p < 0.001
$C_{nitrate}$ (ml/min)	14.9 ± 7.09	25.3 ± 10.1	p < 0.0001
Urine Volume 24h ml)	1712 ± 647	1906 ± 842	p = 0.25
Nitrite			
Plasma (μM)	0.39 ± 0.33	0.25 ± 0.19	p = 0.04
Saliva (μM)	144 ± 94	173 ± 211	p = 0.76
Saliva ratio	0.53 (0.32-0.94)	0.53 (0.29-1.2)	p = 0.70

4.3 Study III

4.3.1 Adherence and efficacy of mouthwash

Adherence to protocol was tested with saliva samples and with an oral bacterial nitrate reducing capacity test (Figure 5). The subjects rinsed their mouth with sodium nitrate (10 ml, 10 mM) for 5 min. The solution was collected and nitrite and nitrite content was measured to examine how much of the nitrate had been converted to nitrite during the 5 min period. Salivary nitrate was significantly increased after mouthwash compared to placebo ($p = 0.0029$), while salivary nitrite was reduced ($p = 0.0008$), which confirmed excellent compliance and efficacy of the mouthwash intervention. Also, salivary nitrite levels accumulating during the oral bacterial nitrate reducing capacity test were significantly lower after mouthwash ($p = 0.0003$). No differences in plasma nitrate and nitrite or nitrate excretion during 24h was observed.

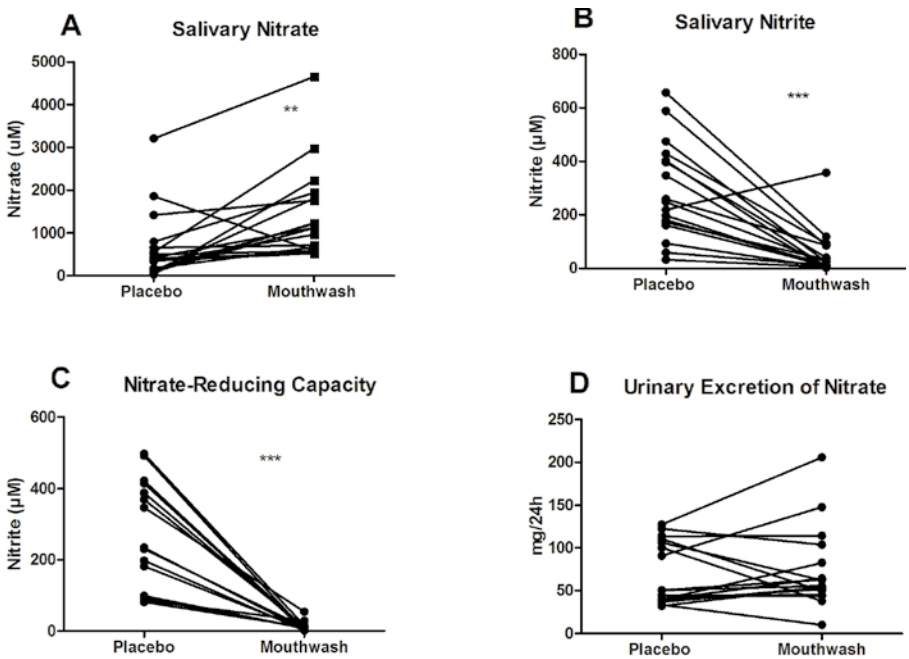

Figure 5. Saliva nitrate (A), saliva nitrite (B), oral bacterial nitrate-reducing capacity (C) and 24 h urinary excretion of nitrate (D) after 3 days of placebo mouthwash compared with 3 days of antibacterial mouthwash. Normally distributed data were analysed with paired t-tests and non-normally distributed data with Wilcoxon matched-pairs signed rank test. Values are presented as means ± SDs

4.3.2 Resting metabolic rate

The primary outcome RMR was not affected by mouthwash treatment being almost identical after mouthwash treatment compared to placebo (1089 vs 1088 kcal/day). Moreover, mouthwash did not alter respiratory exchange ratio. These results indicate that mouthwash does not affect RMR or RER in healthy women (Figure 6). The time-point of measurement was adapted to be on the same days of the menstrual cycle together with temperature measurements. Indeed, both inter-individual differences in RMR during the menstrual cycle (12 %) and intra-individual to range between 2-10 % (mean 4.6 %) has been reported (80). The intra-individual difference in RMR in women seems to be larger than in men (2-4%) (81) and to use a similar time point in the menstrual cycle is therefore essential for reliable comparisons of treatments in intervention studies. In a subsequent study on RMR in vegetarians and omnivores the authors reported a reduction in nitrate-reducing bacteria in both groups (≈ 17 %) after 1 week of antibacterial mouthwash but no difference in RMR in either group (82). In conclusion, blocking NO generation from endogenous nitrate does not seem to affect oxygen demand or energy expenditure as has been reported after nitrate supplementation (69).

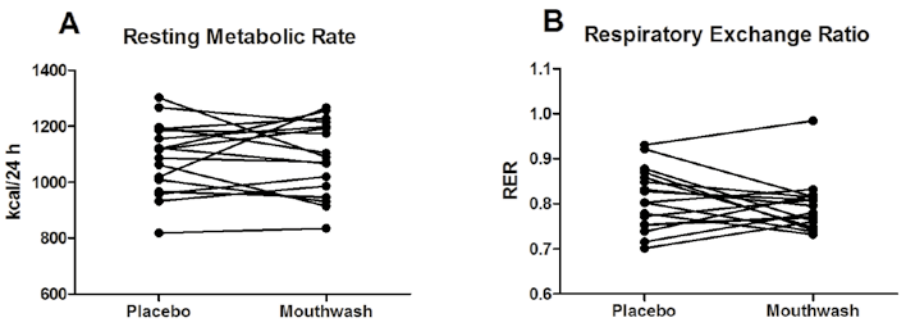

Figure 6. Resting metabolic rate (A) and respiratory exchange ratio (B) after 3 days of placebo mouthwash compared with 3 days of antibacterial mouthwash. Normally distributed data were analysed with paired t-tests and non-normally distributed data with Wilcoxon matched-pairs signed rank test. Values are presented as means ± SDs

4.3.3 Blood pressure

Ambulatory- or office BP did not change after antibacterial mouthwash use compared to placebo mouthwash. These results are supported by the earlier mentioned study by Ashworth et al. on vegetarians and omnivores (82). However, in another study one week of antibacterial mouthwash increased SBP with 3mmHg (33). Moreover, Bondonno et al. reported a 2.3 mmHg SBP increase after 3 days use of antibacterial mouthwash in hypertensive men and women (59). In a study by

Woessner et al. different strengths of mouthwash were used before a nitrate load and chlorhexidine/antibacterial but not antiseptic rinse eliminated the beneficial effect on SBP compared to a control (water) (83). In addition, SBP during treadmill walking was higher after chlorhexidine use followed by a nitrate dose compared to a control rinse (84).

Baseline office BP in the women in Study III was normal (< 110mmHg SBP and < 80 mmHg DBP) compared to the subjects used in the study by Bondonno et al. It is possible that young females in the current study and the hypertensive subjects differ in their abilities to upregulate endogenous NO formation in response to inhibition of the nitrate-nitrite-NO pathway, although a cross talk between these NO pathways has so far only been demonstrated in rodents (85).

4.4 Study IV

4.4.1 Adherence and levels of nitrite and NO

In Study IV 15 healthy men were recruited to take placebo pills or esomeprazole prior to a bolus dose of sodium nitrite (0.3 mg/kg). BP measurements and blood sampling were performed at baseline and 2 h following intake. Plasma nitrite peaked to a similar degree in both groups after sodium nitrite intake, which indicates that uptake was not significantly altered by esomeprazole. Interestingly, nitrite levels decreased more rapidly in the placebo group. The effect of esomeprazole treatment on gastric acidity was indirectly evaluated in three subjects by measurement of NO levels in expelled air after sodium nitrite. Gastric NO formation from nitrite is strictly pH dependent so NO levels can to some extent be used as an indicator of acidity (16). Regurgitation of air was stimulated by drinking carbonated water. Indeed, in the control situation the mean expelled NO after nitrite was almost 600 ppb and after intake of esomeprazole it was < 100 ppb indirectly confirming an increase in gastric pH. In the second part of the study sodium nitrite at three different doses (1, 10 and 30 nmol/kg/min for 10 min) was infused intravenously in 8 healthy volunteers and blood pressure was measured at the end of each dose. Plasma nitrite reached 1.8 μM after the highest dose.

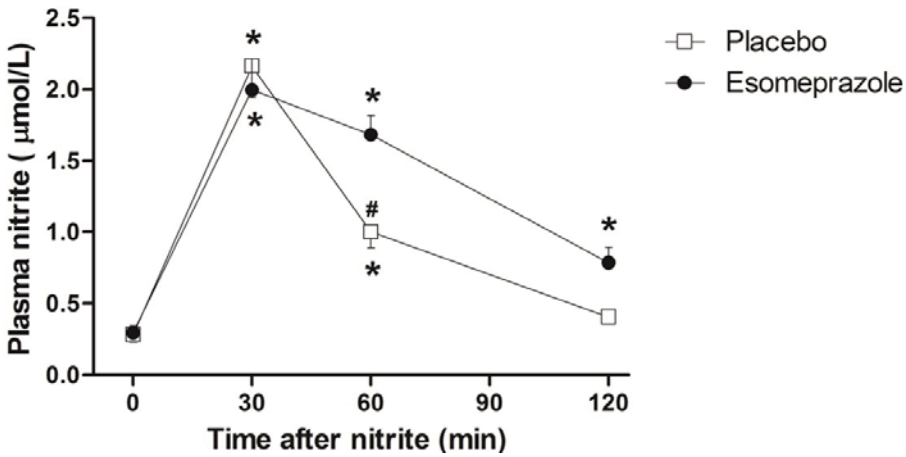

Figure 7. Plasma nitrite changes after ingestion of sodium nitrite. Plasma nitrite levels were higher at 60 min after esomeprazole compared with placebo treatment. Two-way repeated-measures ANOVA with Bonferroni post hoc test was used for between group comparisons (# $p < 0.05$). Within group comparison with baseline (0 min) was performed with repeated measures ANOVA and Dunnett multiple comparison test (* $p < 0.001$). Data are shown as mean ± SEM.

4.4.2 Esomeprazole abolishes the effect of nitrate on blood pressure

Blood pressure was not altered at baseline after intake of esomeprazole. When nitrite was ingested after placebo pre-treatment SBP was reduced by -6 mmHg at 15 minutes. This nitrite-induced reduction was blunted after intake of esomeprazole. A significant difference in SBP response between the two interventions was sustained for an additional 30 minutes. There was no difference in DBP with or without intake of esomeprazole during the 2 h follow up.

In part two of Study IV, nitrite was infused intravenously, but no significant effect on BP was observed despite nitrite in plasma reaching similar levels to the ones seen after oral nitrite administration. These findings suggest that bio-activation of nitrite occurs in the acidic environment of the stomach. The exact mechanisms for this activation, the identity of the final mediator for blood pressure reduction and the physiological role are not clear, but a protective role of maintaining a low gastric pH is supported by the association between increased risk of CVD and chronic use of proton pump inhibitors (86).

Contrary to Study IV, other groups have reported a BP reduction after sodium nitrite infusion, albeit with higher doses than those used in the current study (87, 88). As an example Cosby et al. reported increased forearm blood flow after infusing nitrite (0.36 μmol/min and 36 μmol/min) in healthy subjects (89). Moreover, the robust reduction in SBP (-10 mmHg) following nitrate intake in the study by Webb et al. was seen when nitrite plasma levels were peaking (54). Still, in that study plasma nitrite was < 1uM and thereby around half of that seen in Study IV. It indicates that oral ingestion is needed for bio-activation of nitrite (and possibly nitrate) with a subsequent BP effect. If this is true, earlier identified systemic pathways where nitrite is bio-activated by deoxygenated haemoglobin or xanthine oxidase, would be of minor importance, at least for the acute BP reduction seen after nitrite or nitrate intake.

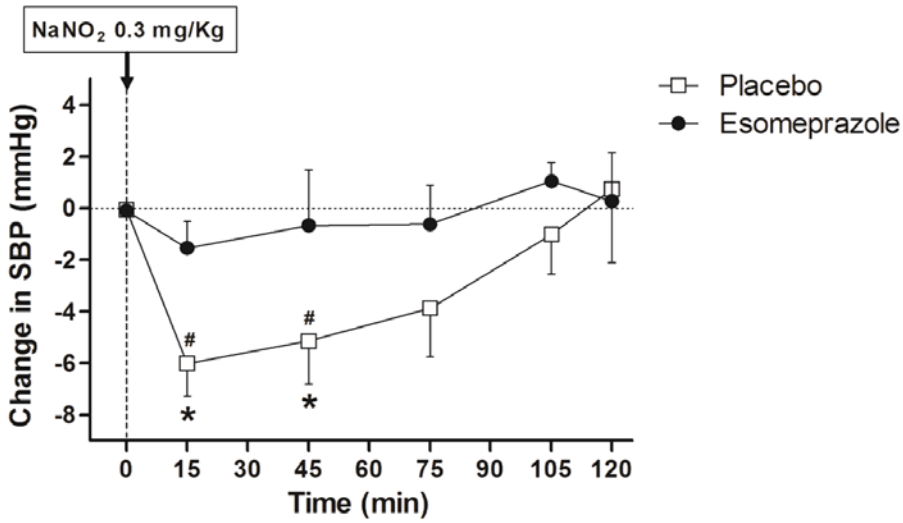

Figure 8. Blood pressure lowering effect of orally ingested sodium nitrite. Esomeprazole (40 mg) or placebo was taken at 3 separate time points (16, 8 and 1 h) before ingestion of sodium nitrite. Sodium nitrite ingestion was followed by a decrease in SBP after placebo pre-treatment but not after esomeprazole treatment. Two-way repeated-measures ANOVA with Bonferroni post hoc test was used for between group comparisons (# $p < 0.05$). Within group comparison was performed with repeated measures ANOVA and Dunnett multiple comparison test (* $p < 0.05$). Data are shown as mean ± SEM.

5 CONCLUSIONS

- Dietary supplementation with leafy green vegetables or pills containing the same amount of inorganic nitrate does not decrease ambulatory SBP in subjects with elevated blood pressure.
- Basal urinary nitrate concentration, fractional excretion of nitrate and 24 h nitrate excretion differs significantly between women and men.
- Antiseptic mouthwash during 3 days does not alter resting metabolic rate or blood pressure in healthy young females.
- The acute blood pressure lowering effect of oral nitrite is abolished by a proton pump inhibitor.

6 FUTURE PERSPECTIVES

6.1 Dietary recommendation for blood pressure regulation

High systolic blood pressure has been identified as the risk factor associated with the greatest burden of disease worldwide (90). This is not surprising given the fact that over 1 billion people worldwide suffer from hypertension and that it is closely linked to increased risk of heart, brain and kidney disease (91). Moreover, from a population perspective a modest reduction in SBP by -2 mmHg can reduce stroke mortality with 10 % and mortality from ischemic heart disease with 5 % (92).

Elevated BP is a result of many factors including an unhealthy diet, physical inactivity, stress, genetic factors etc. Among the environmental factors diet has been pointed out to have the predominant role in BP homeostasis (93). Dietary regimes like the DASH diet has been showed to lower SBP by -5.5 mmHg and DBP by -3 mmHg (94). Also, a vegetarian diet is associated with lower BP (95). Alone, sodium reduction to 2.3g/day can decrease BP with -3.8 mmHg SBP (96). Furthermore, weight control is closely related to hypertension. A meta-analysis concluded that 5.1 kg weight loss reduced SBP with -4.4 mmHg and DBP with -3.6 mmHg in average (97). Evaluation of cost-efficient lifestyle programs with focus on education in dietary habits and weight control is needed for this large patient group.

6.2 Nitrate intake and blood pressure

Inorganic nitrate and the effect on BP have been studied intensely for over a decade. Supplementing with nitrate from natural dietary sources (vegetables) has no known side effects and has the potential of being a safe and cost-efficient alternative to conventional BP pharmaceuticals. In Study I we could conclude that a dose of nitrate achievable through dietary intake could not lower SBP in pre-hypertensive and hypertensive subjects. Future studies need to investigate in which patient group (and responder characteristics) nitrate supplementation could have a clinically relevant effect. It is likely that the BP effect seen after nitrate intake only is evident under very controlled conditions and blunted in studies using out of clinic BP measurements. This has been indicated in a recently published meta-analysis (57).

The pathway for the acute BP lowering effect after nitrate intake has still not been fully elucidated. The role of the acidic milieu in the gastric lumen for bio-activation of nitrite has been discussed in this thesis, but mechanisms for potential signalling pathways via different complexes formed with nitrite or NO, the role of nitrite-reducing pathways, potential altered nerve signalling and possible involvement of vasoactive hormones need to be further investigated.

For future therapeutic use and effective communication with patients, the dose of nitrate needs to be pinpointed. That dose will most likely exceed the current upper limit by EFSA (3.7 mg/kg body weight) (35), for a large part of the population. Recommendations regarding dietary nitrate need to be discussed and re-evaluated with regard to cardiovascular benefits versus potential cancer risk. Several epidemiological studies on fruit and vegetable intake have identified nitrate-rich leafy green vegetables as the food group associated with the greatest cardiovascular protection (43-45). Moreover, ongoing experimental studies show that nitrate and nitrite have a protective effect in ischemia-reperfusion injury models. Interestingly, a long term randomized intervention study on Mediterranean diet and cardiovascular complications reported that the diet did not alter the usual relationship between traditional risk factors (blood pressure, blood lipids etc.), but reduced incidence of CV events independently (98). Study I showed a neutral effect on blood pressure after supplementing with leafy green vegetables for 5 weeks. Future trials should elucidate if dietary nitrate intake could provide a long-term protection against cardiovascular morbidity and mortality through other mechanisms than those related to blood pressure reduction.

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